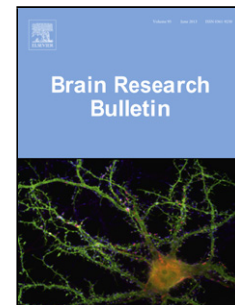


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Differential Gene Expression in the Mesocorticolimbic System of Innately High- and Low-Impulsive Rats

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Abstract

Impulsivity is an important component of many psychiatric illnesses and has been associated with a number of psychiatric disorders such as bipolar disorder and attention deficit / hyperactivity disorder (ADHD). Exploring the different aspects of impulsive behaviour and assigning these to specific neurobiological pathways would advance our interpretation of disorders for which impulsivity is key. Pharmacological studies have implicated a number of neurotransmitters in impulsivity, which in turn have been shown to be affected by several genes in both rodent and human studies of impulsivity. Here, we examine impulsivity-related differences in gene expression in finer detail, using the 2-choice serial reaction time task (2-CSRTT) to assess the molecular signature of impulsivity in brain regions previously linked to impulsive behaviour. Wistar rats were rated as high, (n=6), intermediate, (n=12) or low impulsive (n=6), based on premature responses in the 2-CSRTT, after which RNA was extracted from the nucleus accumbens core (NAcc) and ventral prefrontal cortex (vPFC). RNA from the NAcc and vPFC of high and low impulsivity rats (n=6 per group) was analysed for differential gene expression patterns and exon usage using RNA poly-A tail sequencing. *Pnlsr*, *Mal*, and *Tspan2* were significantly increased in the NAcc of highly impulsive rats, whereas *Ube3a* was significantly decreased. No differences were seen in the vPFC. In addition to changes in gene expression, *Tspan2* displayed differential exon usage in impulsive rats, while functionally, gene expression changes were related to membrane depolarisation and changes in exon usage were linked to sphingolipid breakdown. The changes in gene expression and exon usage observed in this study represent an important step towards defining the molecular architecture of impulsivity. This study therefore represents an important starting point for analysis of the biological role of impulsivity in addiction and other neurological conditions associated with impulsive phenotypes.

Keywords

Impulsivity; Nucleus Accumbens Core; RNA-Sequencing; Genomics; Ventral Prefrontal Cortex

Introduction

Impulsivity is a key component of several neurophysiological disorders such as attention deficit / hyperactivity disorder (ADHD), bipolar disorder, personality disorders, and substance abuse [1] and it is defined as a tendency to act with limited forethought or deliberation [2]. This has multifaceted causes and consequences, including inadequate sampling of sensory information (attentional impulsivity), failure of motor inhibition (impulsive action) or the tendency to accept short-term rewards as opposed to well-informed delayed decisions [3]. Impulsivity may also be linked to intolerance to the frustration of an unavoidable waiting [4]. Given its links to neuropsychological dysfunction, an enhanced understanding of the neural correlates of impulsivity is critical for improving quality of life in a diverse range of clinical populations, as well as highly-functioning individuals.

The mesocorticolimbic reward pathway, with projections from the ventral tegmental area to the prefrontal cortex (PFC), hippocampus, amygdala and the striatum [5], is crucial to impulsive behaviour [2]. Of note, the ventral region of the PFC (vPFC) plays an important role in the inhibition of impulsivity [6]. Conversely, the nucleus accumbens (NAc) within the striatum is predominantly involved in the acquisition and expression of the immediate “wanting” component of impulsivity [7]. Wanting in relation to impulsivity refers to both craving to consume and the effort required to acquire a reward [8, 9]. Within the NAc, the nucleus accumbens core (NAcc) represents a structure where the biological basis of impulsive decisions has been located, as selective lesions of the NAcc are known to increase impulsive tendencies [10] and bias decision-making towards immediate small rewards in a delayed reinforcement choice task in rodents [11, 12].

Dopaminergic innervation of the striatum (including NAcc and shell) is proposed to facilitate behaviours such as impulsivity, with the shell being particularly responsive to the reward from drug abuse [13], whereas the NAcc is reactive to response-reinforcement learning [14]. Therefore, it is not surprising that functional variants of genes involved in regulating dopaminergic activity such as dopamine receptor D4 (*DRD4*) are altered in impulsive individuals [15]. In humans, *DRD4* contains a

variable number of tandem repeat (VNTR) polymorphisms that range from 2 to 11 repeats across healthy individuals, with those expressing at least one 7-repeat allele exhibiting alterations in inhibitory control [16, 17]. Absence, or a reduced expression, of *Drd4* in mice results in impaired response inhibition, a feature of impulsivity, in the 5-choice continuous performance test [18].

In addition, variants of the dopamine activated transporter (*DAT1* or *SLC6A3*) gene have also been implicated in ADHD and other impulsivity-related disorders [19, 20]. *Dat1* knock-out mice have a marked hyperlocomotion and an impaired cliff avoidance reaction [21]. Moreover, rats overexpressing *Dat1* in the NAc show increased impulsivity and inclination towards risk [22]. In humans, impulsivity is relatively heritable with approximately half of the variance in impulsivity explained by genetic factors [23], which is similar to many of the conditions associated with increases in impulsive behaviour such as ADHD and addiction [24]. We hypothesised that genes relevant to neuronal structure, including synapse development, neuronal membrane integrity and myelination would be altered in highly impulsive rats.

Impulsivity is a complex behavioural construct and animal models have facilitated the deconstruction of the distinct entities of this behaviour. The 2-choice serial reaction time test (2-CSRTT) is a test adapted from human studies that provides an experimental framework to observe impulsivity via independent measures of attention and motor impulsivity [25]. In this test, rats are presented with two levers that can be pressed, representing the two choices for action. On each trial, a stimulus light is illuminated above one of the levers, indicating that rats can earn a reward by pressing that specific lever [25]. Lever pressing behaviour before the onset of the light reflects a failure of behavioural inhibition and is classified as premature responding, providing an index of impulsive behaviour [2, 26]. The difficulty of the task increases over the course of a session (typically 100 trials) by reducing the presentation time of the stimulus light, allowing assessment of behaviour across a range of levels of impulsivity. This model also provides a sound translational framework to consider the specific genes (and their relevant neurotransmitters and proteins) which control impulsivity [27].

In the present study, we investigated the genomic architecture of the mesocorticolimbic pathway, specifically the NAcc and vPFC, in innately low- and high-impulsive rats which were identified based

on their performance in the 2-CSRTT. These data provide essential and novel insight into the neurobiological basis of impulsivity, furthering our understanding of the pathophysiology of these disorders and the development of novel treatment strategies.

2

Materials and methods

2.1

Rats and behaviour

24 male Wistar rats (250-300 gram) were purchased from Harlan, UK. Animals were group-housed (4 per cage) in a facility maintained at $21 \pm 1^\circ\text{C}$, with $55 \pm 10\%$ humidity, on a 12h:12h light:dark cycle with lights on at 7.30 am. Animals were food restricted to maintain 90% of their initial bodyweight, receiving 11 grams of chow per day per rat. All experiments were approved by the Animal Experimentation Ethics Committee at University College Cork according to the European Directive 86/609/EEC and Recommendation 2007/526/65/EC.

2.1.1

2-Choice serial reaction time apparatus

The apparatus consisted of an extra tall medium density fibreboard sound-attenuating cubicle with a standard modular test chamber. Test chambers had a stainless steel grid floor, a house light, two stimulus lights (1-inch white lens), two standard response levers, a modular pellet dispenser (45 mg) and pellet receptacle (Med-Associates, USA).

2.1.2

2-Choice serial reaction time task training

Animals were trained according to a protocol described by [25], consisting of three pre-training habituation sessions and four phases of training (**Figure 1**). During habituation, rats were first given 100 5TUL sucrose pellets (1811155, Testdiet, USA) under a 15 second schedule of reinforcement. The

session ended when all pellets had been consumed. Next, animals were shaped to press a lever to receive the sucrose pellet from a trough that does not project into the chamber. In the final habituation session, animals could earn half of the pellets (50) by pressing one of the levers and the other half by pressing the other lever. The levers were not retracted after the maximum number of pellets per lever were earned.

Throughout all subsequent training phases, rats underwent training in 30-minute sessions, with one session per day. A trial started with the illumination of the cue lights situated above the levers and ended after the rat responded by pressing a lever or, in Phase 3 only, after a specified time period. A correct response, defined as a lever press under the illuminated cue light within the specified time frame, resulted in delivery of a sucrose pellet. A correct response initiated an inter-trial interval (ITI) of 5 seconds, wherein only the house light was on. An incorrect response was defined as a lever press other than the illuminated cue light, while the failure to press any lever during a trial was designated as an omission. Both omissions and incorrect responses were followed by a time-out period of 5 seconds, wherein the house light was turned off until initiation of the next trial. If an animal pressed the lever during an ITI or time-out period, a premature response was counted [25], and a new 5-second time-out period started.

During Phase 1 of training, both cue lights were illuminated on each trial and rats earned a sugar pellet by pressing either lever. When the rat earned 100 food pellets within a 30-minute session, the second training phase commenced. In Phase 2, only one cue light was illuminated for each trial, in a pseudorandom order, and rats earned food rewards by pressing the lever specifically associated with the illuminated cue light. Phase 2 continued until the rat earned 100 pellets within a 30-minute session. Phase 3 was similar to Phase 2, except that trials were time-limited such that the cue light remained illuminated for a maximum of 36 seconds. Rewards were delivered if rats pressed the appropriate lever during the illumination period or within 2 seconds of the extinction of the cue light. Rats could earn a maximum of 100 food pellets in each of these sessions. Once the maximum number of rewards was reached within a session, the duration of cue illumination (i.e., the length of each trial) was systematically decreased across subsequent sessions (from 36 to 16, 8, 4, 2, 1.5, then finally 1 s) which

varied from animal to animal. Training was considered complete when animals reached a stable baseline performance (accuracy >80% correct choice and <20% errors of omission) for five days, as described by [28]. Once this threshold of performance was achieved, 2-CSRTT training was stopped and animals were fed *ad libitum* for two weeks to negate the effect of food restriction and the 2-CSRTT.

Four measures were recorded to assess task performance (**Figure 2**): (1) Premature responses (i.e. number of lever presses during the ITI). This measure is the most relevant to behavioural inhibition and impulsivity as an increased number of premature responses reflects increased motor impulsivity [26, 29]; (2) Correct latency (i.e. the time between onset of stimulus and a correct response); (3) Errors of omission (i.e. the number of trials during which no response was made during stimulus presentation or within the limited hold); (4) Response accuracy (i.e. the ratio correct to incorrect responses), used as an index of attention, as described previously [25].

Animals were rated either low, medium or high impulsivity (6, 12 and 6 rats respectively), based on the mean number of premature responses over the last 5 training sessions, as described in [7]. Specifically, rats within the lower quartile were labelled low-impulsive and rats within the upper quartile were labelled high-impulsive, these rats were selected for further analysis, samples from rats with intermediate impulsivity were not analysed further (**Table 1**). Rats were sacrificed by decapitation between noon and 2 pm, whole brains were snap-frozen using ice-cold isopentane and stored at -80°C.

2.1.3

Brain Micro-punching

All materials were cleaned using RNazap prior to use and subsequently placed in the cryostat (-20°C) along with whole brains 20 minutes prior to punching to acclimatise to the temperature. Snap-frozen brains were placed on a steel matrix (Steel Brain Matrix, 1.0mm section size, Coronal, 175-300g (Stoelting, 51388) and secured using Tissue-tek (VWR, 25608-930). Blades (Fisher Scientific, 11904325) were used to slice the brain, after which the brain regions of interest were punched using a needle (BD Microlance 3 Needles Cream 19g x 1.5" x 100. The inner diameter of this needle is 0.686mm), using the rat brain atlas as a guide [30]. For the vPFC, we specifically isolated the prelimbic

cortex, medial orbital cortex, and cingulate cortex subregions. Brain punches were subsequently placed in an RNase-free Eppendorf and stored at -80°C for mRNA isolations.

2.2

Total RNA isolation and RNA Sequencing

All work surfaces and materials were cleaned with RNase Zap spray (AM9780, Ambion). Total RNA was isolated with the mirVana™ miRNA Isolation Kit as per the manufacturer's instructions. RNA concentration was assessed with the Nanodrop ND-1000 (Thermo scientific) and quality of the RNA was examined with the Agilent RNA 6000 Nano Chip using the Bioanalyzer (Agilent Technologies). vPFC and NAcc samples from rats with a high impulsivity score (n=6) or low impulsivity score (n=6) were sequenced. Library preparation and sequencing, as well as Fastq-file generation, was done by Beckman Coulter Genomics (Danvers, MA, USA). Paired-end reads of 2×100 base pairs were produced on an Illumina HiSeq2500 sequencer.

2.3.1

Bioinformatic analysis pipeline

Quality control and mapping to reference genome

Fastq-format reads were quality filtered and trimmed using Trimmomatic (v0.32), [31] with the following non-default parameters: AVGQUAL: 20; SLIDINGWINDOW: 4:20; LEADING: 10; TRAILING: 10; MINLEN: 60. Alignment to the mouse reference genome (GRCm38.p3) was achieved using the STAR aligner (v2.4.0f1) [32], with default options and an index compiled with gene models retrieved from the Ensembl database (release 78).

2.3.2

Differential gene expression and functional enrichment analyses

Ensembl database release 78 gene models were also used for counting mapped reads per gene using HTSeq-Count (v0.6.0) [33], with the following non-default parameters: -s: no; -r: pos; -q -f bam -m

intersection-nonempty. Differential gene expression was calculated for pairwise comparisons using the DESeq2 R-package (v1.6.2) [33], with default parameters. Genes with an FDR-adjusted p-value ≤ 0.1 were considered differentially regulated. These genes were used for hierarchical clustering of all samples using the gplots R-package on HTSeq-generated counts for each sample.

2.3.3

Differential exon usage analysis

For detection of differential exon usage, the DEXSeq R-package was used (v1.12.2), [34] with default parameters. The FDR-corrected p-value significance level was set to 0.1. Ensembl database release 78 was used to provide exon models. Exon location was assessed based on the location of the exon in 5' untranslated, 3' untranslated or within a translated region using Ensembl release 78 transcript models for rats.

2.3.4

Quantitative real-time PCR (qRT-PCR)

All qRT-PCR experiments were performed using specific PrimeTime® qPCR assays (Integrated DNA Technologies, Table 3). cDNA from the high and low impulsivity rats was synthesised using the high capacity cDNA reverse transcription kit (Applied Biosystems) and diluted to 50 ng/μL. qRT-PCR was performed with PrimeTime® Gene Expression Master Mix in 3 technical replicates for each biological sample (n=6 per group) on the Roche LC480 384 well system, fold differences of mRNA levels were normalised against the geometric mean of two reference genes (*Actb* and *B2m*), analysed using the $2^{-\Delta\Delta Ct}$ method [35] and presented as fold change relative to the low impulsivity group (n=6).

2.5

In-silico Analysis

The overlap between gene expression lists was plotted using Venny [36]. Enrichment analysis of mRNA gene sets and alternatively spliced gene sets were analysed using ENRICHR. Transcription factor enrichment was analysed using ENRICHR [37], exon location was manually curated using the Ensembl

genome browser. The significance of the ENRICHR analysis is calculated using three types of enrichment score. These tests are 1) the Fisher exact test, 2) the z-score of the deviation from the expected rank by the Fisher exact test, and 3) a combined score that multiplies the log of the p-value computed with the Fisher exact test by the z-score. The length of the bar in the figures is determined by this final statistic (log of the p-value X z-score).

2.6

Statistics

Statistical analyses were conducted using SPSS software, version 24 (IBM Corp., Armonk, NY). Data are presented as mean \pm standard error of the mean or mean \pm standard deviation where stated, an unpaired two-tailed t-test and a one-way analysis of variance (ANOVA) were used to analyse the difference between groups. Statistical analysis of differential exon usage is described further in [34]. A *p* value of < 0.05 was deemed significant in all cases, false discovery rate analysis was performed using Benjamini–Hochberg correction testing following which significance was calculated at $q < 0.1$.

Results

3.1 Baseline behaviour in the 2-choice serial time task test (2-CSRTT)

Rats were assigned to one of 3 groups based on the percentage of premature responses made in the last 5 sessions of training (**Figure 2G, H, Table 1**), as previously described [7]. In total, 50% of rats (n=12) had an intermediate number of premature responses (mean $16.78\% \pm 3.43$, range 12-22.60%), 25% of rats (n=6) displayed a low number of premature responses (mean $9.77\% \pm 2.29$, range 5.20-11.20%), while 25% of rats (n=6) accumulated a high number of premature responses (mean $33.30\% \pm 8.72$, range 24.60-48.40%) (**Table 2**). Additional measures from the 2-CSRTT – correct latency, accuracy and the number of omissions – were also recorded (**Figure 2A-F, Supplemental Figure 1 A-H**). When animals were separated into percentiles based on these measures it was clear that there was no significant overlap between scores for premature response and other measures from the impulsivity task.

3.2 Gene Expression and Impulsivity in the NAcc and vPFC

We hypothesised that there would be differences in gene expression as a function of impulsivity. Therefore, we sought to identify changes in gene expression in the NAcc and vPFC. RNA sequencing the vPFC and NAcc of rats with a low impulsivity rating (n=6), and a high impulsivity rating (n=6), was performed and a genome mapping rate of 85% achieved. Principal component analysis of sample distribution by transcript abundance revealed a clear regional separation between gene expression in the NAcc and the vPFC. Regional differences in gene expression accounted for 62% of the variation in transcript abundance. In addition, a separation based on impulsivity was also observed in the NAcc (**Figure 3A**), explaining a further 11% of the variance. In total, 22 genes in the NAcc (8 upregulated and 14 down regulated) were differentially expressed between low and high impulsivity rats (with a *P* value of < 0.1 following adjustment for multiple comparisons; **Figure 3C**). No significant impulsivity-related gene expression changes were noted in the vPFC.

3.3 Functional Enrichment of Gene Expression Changes in the NAcc

Significantly different genes from the NAcc were examined for enrichment in gene sets relative to biological processes for overrepresentation. Functional enrichment for genes important to membrane depolarisation and sodium transport were significantly overrepresented in impulsive rats (**Figure 3D**). Moreover, to provide insight into the upstream processes involved in the NAcc differences, we performed *in-silico* analysis for transcription factors that bind to the genes significantly altered in impulsive rats. Using ChIP-seq data from the ENCODE project and ChIP-X enrichment analysis (ChEA) gene set library project, we determined the transcription factors *Ubt* and *Tcf3* were significantly enriched at the promoters of genes changed in the NAcc of impulsive rats (**Figure 3E**, **Table 7**). Using average linkage clustering, the z-score for RNA-Seq reads for those genes significantly altered between low and high impulsivity rats provided a clear clustering between these two groups (**Figure 3F**).

3.4 RT-qPCR of RNA-seq Results

Given the subtle but distinct changes in gene expression between impulsivity groups, we sought to further confirm this by qRT-PCR in the same NAcc samples. Six genes (*Pnlsr*, *C3md3*, *Mal*, *Tspan2*, *Ube3a* and *Cplx1*, **Table 3**) were selected for further validation based on statistical differences in fold change and biological relevance. *Pnlsr*, *Mal*, and *Tspan2* were significantly increased in the NAcc of highly impulsive rats while *Ube3a* was significantly decreased with *Cplx1* showing a similar trend. *C3md3* expression was not different between groups (**Figure 4 A-F**).

3.5 Alterations of the Genomic Architecture of Impulsive Rats

Alternative splicing is a process by which gene expression may be regulated and is regularly observed in the brain [38, 39]. We have observed changes in gene expression via two different methods in the NAcc and to further verify the genomic architecture of the impulsive brain we analysed our dataset for differential exon usage in the vPFC and NAcc of low and highly impulsive rats. Similar to our gene expression data, no changes in exon usage were noted in the vPFC. Interestingly, 45 genes showed

changes in exon usage in the NAcc (**Figure 5, Table 4**), with *Hnrnpdl* and *Tspan2* (**Figure 5B**), being present in both gene expression and exon usage readouts and 6 genes having more than one site of differential exon usage (**Table 4**). Usage of exons can affect all parts of a transcript, including the 3' untranslated region (3' UTR) [40], with transcripts in the brain containing much longer UTRs than other tissues [41]. We found that 45% of exons were located in translated regions while the remainder were located in the 3' UTR or 5' UTR regions, which may indicate how different exon usage may impact on the post-transcriptional regulation of gene expression in the brain (data not shown).

Transcription factors represent an additional critical control point in gene expression and the genomic landscape. To integrate our data from gene expression and exon usage with the transcriptional machinery we examined transcription factor enrichment in our list of genes from impulsive rats with differential exon usage in the NAcc. Overall, the transcription factors *Runx1*, *Ubtf*, *E2f6*, *Tcf7l2* and *Creb1* were significantly over-represented (**Figure 5D, Table 5**), indicating a further level of complexity in the genomic architecture of the NAcc in impulsivity. Having demonstrated changes in gene expression, exon usage and predicted transcription factor use, we wanted to determine a predicted functional enrichment for the NAcc in which exon usage is increased (or decreased). To this end, we used ENRICHR to analyse functional enrichment of genes with altered exon usage in the NAcc of impulsive rats (**Figure 5C, Table 6**). Interestingly, there was significant over-representation of genes associated with pathways related to the excitatory synapse, the biogenesis of protein complexes, hydrolase activity and filament length suggesting that in addition to changes in gene expression, these pathways may be implicated in the biological process underpinning the NAcc in impulsive rats. Moreover, genes with altered exon usage were also significantly over-represented in impulsive rats, suggesting that genes associated with Alzheimer's progression and pathogenesis may also be involved in the neurobiology of impulsivity.

Discussion

Determining the transcriptional landscape of the circuits involved in impulsivity will aid in a better understanding of the underlying biology of this behaviour. In this study, we find evidence of significant changes in gene expression specific to the core of the nucleus accumbens (NAcc). We examined the NAcc and the ventral prefrontal cortex (vPFC), two brain regions associated with impulsivity, to assess molecular variances between these distinct regions that may imply differing function associated with impulsivity. Surprisingly, no differences were noted in the vPFC. Conversely, rats with high and low impulsivity displayed consistent and distinct differences in the NAcc in terms of gene expression (evaluated using 2 different methods), exon usage and finally, significant involvement of relevant molecular and biologically meaningful pathways.

This study shows changes in gene expression in the NAcc of rats two weeks after undergoing the 2-CSRTT, where animals were classified in an unbiased manner into groups based on their impulsivity score in this food-based reward task. Experimental methods that induce impulsive behaviour in rodents, either pharmacologically or through lesions of selective brain regions, have proven the contribution that specific brain regions and their associated neurotransmitters have made to impulsive behaviour [27]. However, animal models that depict the fundamental naturalistic characteristics of impulsivity have advantages in terms of face and construct validity compared to those using pharmacological approaches or brain-region specific lesions.

In this study, latency, errors of omission and accuracy improved throughout the 2-CSRTT. While the number of premature responses also decreased across the task, rats did not eliminate premature responses completely and the percentage of premature responses thus provided a clear, unbiased representation of impulsivity. Although additional measures from the 2-CSRTT were recorded, we focussed our analysis on the percentage of premature responses as a measure of impulsivity as previously reported [25, 42]. Moreover, accuracy in the 5-CSRTT has been shown to be unaffected by lesions to the NAcc thus we did not incorporate this into our molecular analysis [43]. In addition, given

the 2-CSRTT has only 2 levers, rats will always have a 50% chance of guessing correctly, whereas this chance is only 20% in the 5-CSRTT thus making this an unsuitable measurement in the 2-CSRTT. Sequencing the transcriptome of rats with high and low impulsivity, somewhat surprisingly, revealed limited changes in gene expression or exon usage in the vPFC. Impulsivity consists of both an impulsive action and an impulsive choice [2], of which the former is evaluated using the 2-CSRTT. There is an acceptance that neural substrates and dopamine function differ between these two trait identities [7], and it has also been suggested that in models of addiction (both nicotine and cocaine) a disparity exists between these subregions relative to dopamine release. Lack of gene expression changes in the vPFC may be for a variety of reasons. For example, the vPFC may be more reactive to tests that examine impulsive choice such as the delayed reward task [44]. Alternatively, dopamine release in the NAcc, which is increased in impulsive rodents, may inhibit responses in the vPFC, or the vPFC may not respond to stimulation in the 2-CSRTT [7]. The vPFC is known to be important in inhibiting impulsive behaviour which suggests it may be more important in impulsivity relevant to drug addiction.

On the other hand, RNA sequencing of the NAcc revealed significant gene expression changes in highly impulsive rats compared with rodents exhibiting low impulsivity (**Figure 4A-F**). Of these 22 significant genes little is known of their functional relevance to impulsivity. Six of these genes were selected based on statistical significance and fold change relative to low impulsivity rats (**Figure 3C**). Of those genes selected, *Pnlsr*, *Mal*, and *Tspan2* were increased and *Ube3e* was significantly decreased in highly impulsive rats compared with low impulsivity rats confirming these changes from our RNAseq data.

Little is known about the significantly reduced gene *Pnlsr*, as its function is undefined other than that it forms part of protein complexes in corneal epithelial cells [45]. Myelin and lymphocyte protein (*Mal*) was also significantly increased in the NAcc of impulsive rats. *Mal* is a transmembrane-tetraspan proteolipid expressed by oligodendrocytes and Schwann cells where it is a constituent of compact myelin [46]. Over-expression of *Mal* is associated with abnormal myelination and hypomyelination [47]. In humans it has been suggested that a delay in myelination in youths may influence risk-taking behaviour [48]. Similarly, in a rodent model of alcohol addiction, demyelination is observed in the PFC mainly due to the activation of innate immune receptors [49]. Similarly, impulsive rats exhibited

increased expression of the transmembrane protein *Tspan2* which, like *Mal*, is also localised to compact myelin [50], where it plays an important role in oligodendrocyte development and neuroinflammation [51]. Interestingly, *Tspan2* also showed a significant change in exon usage in highly impulsive rats, suggesting that this gene may be a key molecular node in the molecular signalling underpinning impulsive action in the NAcc.

On the other hand, the E3 ubiquitin ligase *Ube3e* was significantly reduced in highly impulsive rats. *Ube3e* is involved in plasticity and synapse development, as well as the degradation of proteins such as *Arc* in the synapse [52]. Due to its role in synaptic biology, it is intriguing to note that synaptic formation, function and plasticity are also altered in attention-deficit hyperactivity disorder (ADHD), a condition noted for excessive risk-taking and risk-seeking behaviour [53]. Strikingly, impulsivity is a behavioural feature in Angelman Syndrome, a neurological disorder caused by mutation of the E3 ubiquitin ligase *Ube3A* that reduces expression of *Ube3A* [54, 55].

Collectively, using pathway analysis, these genes are functionally enriched in pathways predominantly related to sphingolipid metabolism and sphingomyelin. Intriguingly, low levels of sphingolipids have been found in children with ADHD [56], and given the location and function of *Mal* and *Tspan2* it is interesting to hypothesise that myelination may be altered somehow in disorders of impulsivity. The vascular endothelial growth factor (*Vegf*) receptor signalling pathway was also significantly overrepresented in our list of differentially expressed genes. Little is known about the role of *Vegf* in impulsivity, but it has been implicated in ADHD with its expression increased in peripheral blood [57]. To identify further upstream mechanisms in the transcriptional profile, we pinpointed a number of common transcription factors amongst differentially expressed genes, suggesting that sphingolipid metabolism may drive gene expression changes in impulsivity in the brain and that these gene expression changes may be linked to a number of key transcription factors such as *Ubt1* and *Tcf3*.

In addition to altered gene expression, splicing is an important component of genome complexity and contributes to different exon usage and generation of different transcript isoforms [58]. We found 45 genes with significant changes in exon usage in the NAcc, whereas once again no changes were noted in the vPFC. As previously mentioned, only 2 genes were present in both lists comparing gene

expression (*Tspan2* and *Hnrnpdl*), suggesting exon usage and gene expression may influence different signalling pathways. To further examine this hypothesis, we analysed the functional enrichment of these alternatively spliced genes and noted that membrane depolarisation and sodium ion transport were functionally enriched in these gene sets. Moreover, no overlap in these gene sets was noted between alternative splicing and differently expressed genes, suggesting that different exon usage in these biological processes may play an important role in the impulsive NAcc in a manner mechanistically different from differently expressed genes. In addition, it was also noted that a number of transcription factors such as *Runx1* and *Ubtf* were common to those genes with alternatively spliced exons. Interestingly, *Ubtf* was common to pathways, possibly representing a key node in the signalling pathways involved in the impulsive action in the NAcc.

In conclusion, our data illuminates alterations in the transcriptional landscape of the NAcc of highly impulsive rats following a food-based reward task. We show that molecular changes in the NAcc are distinct and consistent. Specifically, changes in gene expression are linked to membrane depolarisation and changes in exon usage are linked to sphingolipids and their breakdown. This study represents an important starting point for further analysis of the biological role of impulsivity in addiction and other neurological conditions associated with impulsive phenotypes such as ADHD.

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Figure Captions

Figure 1 Experimental design and two-choice serial reaction time task outline.

(A) Schematic representation of experimental design using 24 male Wistar rats. (B) The two-choice serial reaction time task (2-CSRTT). **Left:** apparatus, consisting of a chamber with a food dispenser at the rear and at the front of the chamber is two equally spaced holes, each equipped with a light bulb above and a lever directly underneath. **Right:** Possible trial sequences in the task. Rats initiate trials by responding to the food dispenser at the rear; after a 5-second delay, a brief stimulus is presented over a lever. Subjects must respond to that lever (i), (correct response) within a certain time to obtain food. If they respond to the wrong hole (ii), (incorrect response), respond before the stimulus is presented (iii), (premature response), or fail to respond (iv), (omission), they are penalised with a period of darkness before the next trial begins. (C) Overview of the experiment, including training protocol. Training finished on day 15, and on day 17 all rats had completed 100 trials in 30 minutes successfully.

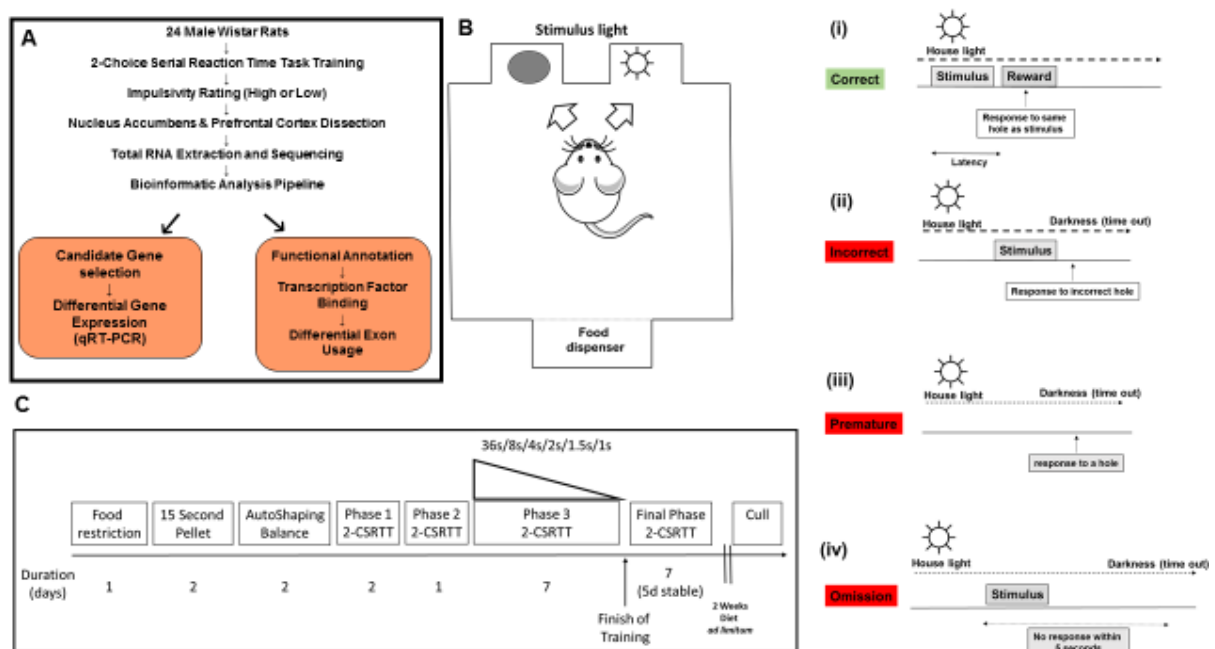


Figure 2 Dependant measures of an impulsivity task performance

(A) Correct latency, the time between onset of the stimulus and a correct response over 22 sessions. (B) Animals were rated either low, medium or high for the correct latency (6, 12 and 6 rats respectively), based on the time between the onset of stimulus and a correct response over the last 5 training sessions *** $p \leq 0.001$, ** $p \leq 0.01$, relative to the high latency group. (C) Accuracy, the ratio of correct to incorrect responses over 22 sessions. (D) Animals were rated either low, medium or high (5, 14 and 5 rats respectively), based on the percentage of correct responses to incorrect responses over the last 5 training sessions *** $p \leq 0.001$ relative to the high correct responses group, * $p \leq 0.05$ relative to high correct responses group. (E) Errors of omission, the number of trials during which no response was made during stimulus presentation or within the limited hold time (F) Animals were rated either low, medium or high (8, 10 and 6 rats respectively), based on the number of omissions over the last 5 training sessions *** $p \leq 0.001$ relative to the high omissions group, * $p \leq 0.05$ relative to medium omissions group. (G) Premature responses, the number of lever presses during the inter-trial interval, as described in [25]. (H) Animals were rated either low, medium or high impulsivity (6, 12 and 6 rats respectively), based on the mean percentage of premature responses over the last 5 training sessions, as described in Table 1 and 2, [7]. $p \leq 0.05$ was considered statistically significant. Differences between low, intermediate and high impulsivity were measured using one-way ANOVA followed by Bonferroni's multiple comparison test. *** $p \leq 0.001$ relative to the high impulsivity group, * $p \leq 0.05$ relative to the medium impulsivity group. (Figure A, C, E, H), Data is represented as the mean and standard deviation (S.D.) over 22 testing sessions, the shaded boxes represent the final 5 sessions of testing. Data are presented as median with interquartile range (IQR) and min/max values as error bars on (B, D, F, G).

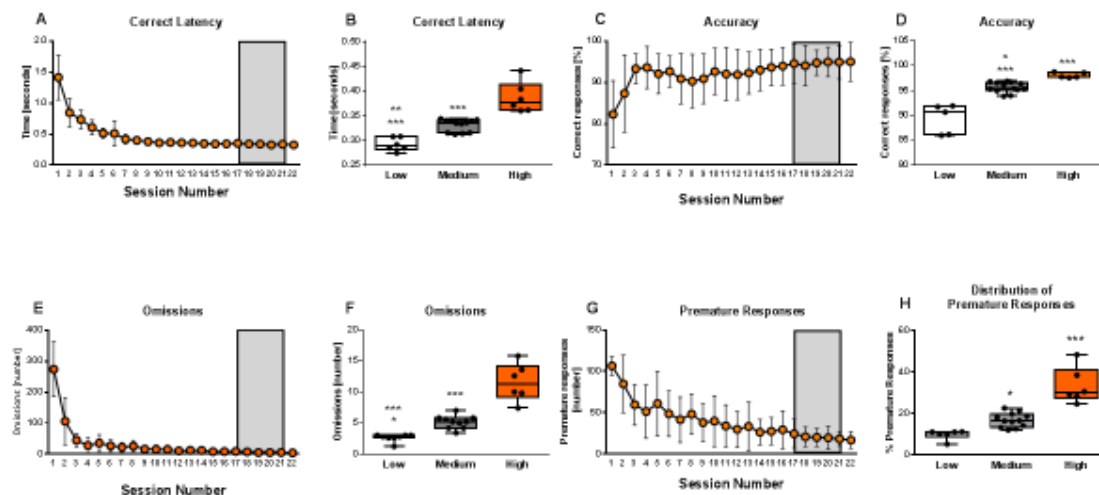


Figure 3 Distinct gene expression changes in the Nucleus Accumbens Core of impulsive rats

(A) Principal component analysis (PCA) of RNA-Seq results, each triangle represents an individual sample from the vPFC while each circle represents a sample from the NAcc. Data in violet and green represent samples from low-impulsive rats ($n=6$), samples in blue and orange represent samples from high-impulsive rats ($n=6$). (B) **Top:** Representative image of the NAcc and ventral PFC regions which were micro-punched for RNA-Seq and analysed for changes in gene expression. **Bottom:** Venn diagram summarising significant gene expression changes between the NAcc and PFC relative to the degree of impulsivity. (C) The log fold change (FC) of genes differentially expressed in highly impulsive rats relative to rats with low impulsivity scores. Changes in expression between groups were measured by comparing the normalised read count for all genes between high and low impulsivity rats in the NAcc and PFC. (D) Functional gene enrichment of biological function from a list of genes with mRNA expression differences in impulsive rats ($n=6$) in impulsive rats. The length of the bar represents the significance of that specific gene-set or term. In addition, the brighter the colour, the more significant that term is. * < 0.05 following adjustment for FDR. (E) Enrichr enrichment analysis of transcription factors which target the list of genes with mRNA expression differences in impulsive rats ($n=6$). Solid red squares represent a transcription factor (top of matrix) which targets the corresponding gene (left of matrix). The significance of this targeting is represented by the length of the red bar on the top row of the matrix, see also Table 7. (F) Hierarchical clustering of z-scores was performed in Genesis (see methods section). P values were adjusted to account for effects of multiple comparison and $p \leq 0.1$ was considered significant for adjusted comparisons.

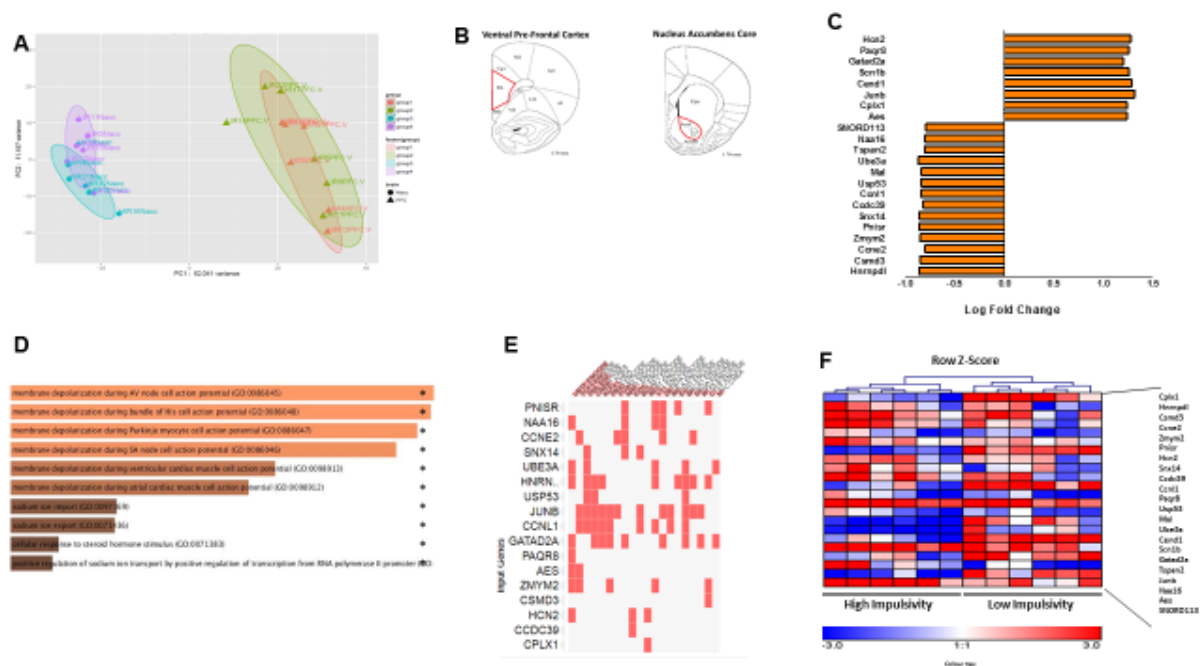


Figure 4

(A-F) qRT-PCR quantification of selected genes altered in the NAcc of impulsive rats (n=6) compared with low impulsive rats. (A) *Pnlsr*, (B) *C3md3*, (C) *Mal*, (D) *Tspan2*, (E) *Ube3a* and (F) *Cplx1*. Fold differences of mRNA levels were normalised against the geometric mean of two reference genes (*Actb* and *B2m*) and were compared to gene expression levels in LIR rats (n=6). . *** $p \leq 0.001$, ** $p \leq 0.01$ and * $p \leq 0.05$, relative to low impulsivity group. Data are presented as mean with standard error of the mean (S.E.M) values as error bars.

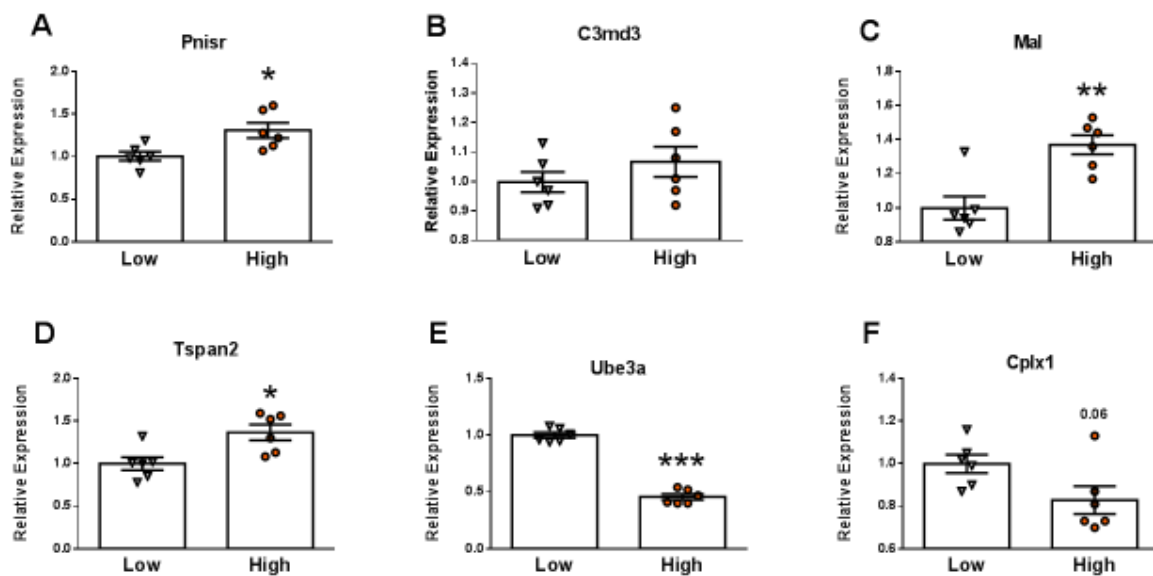
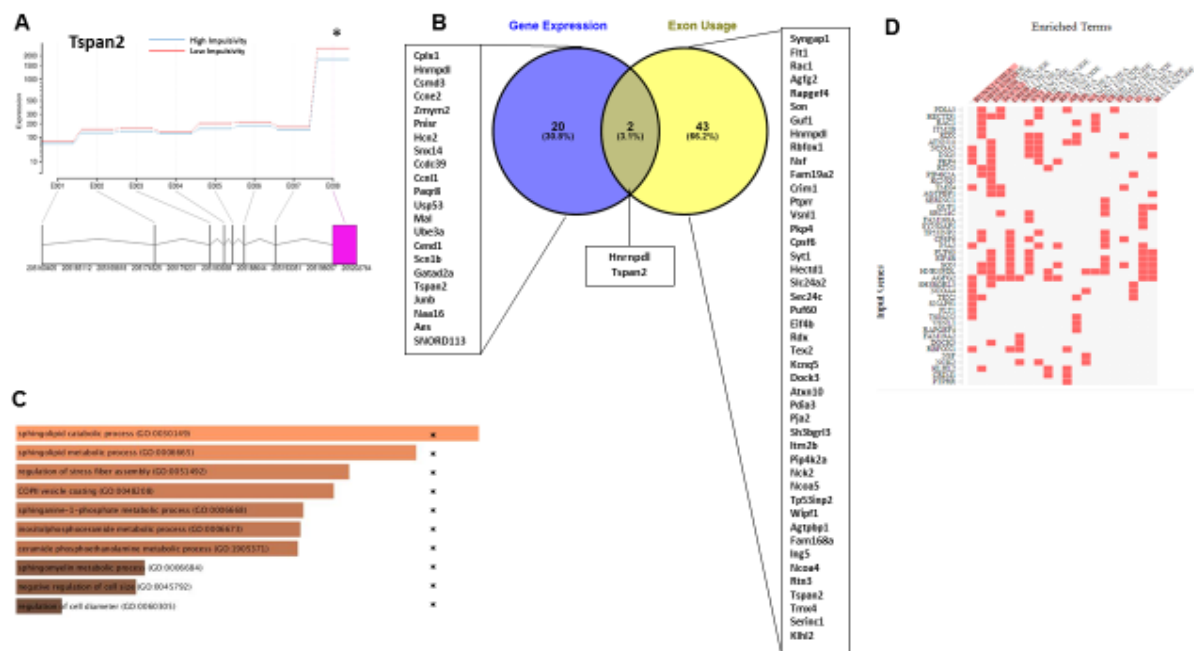


Figure 5

(A) Representative plot showing decreased expression of exon 8, from the gene *Tspan2* in impulsive rats in the NAcc compared with LIR rats, (n=6 per group). (B) Venn diagram summarising overlap between exon usage and gene expression changes with *Tspan2* and *Hnrnpdl*. (C) Gene set enrichment analysis of genes with altered exon usage in impulsive rats. The length of the bar represents the significance of that specific gene-set or term. In addition, the brighter the colour, the more significant that term is. * < 0.05 represents significance following correction for multiple testing. (D) *Enrichr* enrichment analysis of transcription factors which target the list of genes with altered exon usage in impulsive rats (n=6). Solid red squares represent a transcription factor (top of matrix) which targets the corresponding gene (left of matrix). The significance of this targeting is represented by the length of the red bar on the top row of the matrix, see also **Table 8 (E)**.



Tables

Table 1: Percentage (%) of premature responses in the last 5 sessions of the 2-CSRT test.

Rat #	Session 18	Session 19	Session 20	Session 21	Session 22	Mean \pm S.D
1	11%	13%	18%	13%	16%	14.2 \pm 2.8%
2	14%	13%	17%	24%	14%	16.4 \pm 4.5%
3	21%	8%	9%	14%	8%	12.0 \pm 5.6%
4	33%	32%	51%	36%	41%	38.6 \pm 7.8%
5	10%	5%	3%	2%	6%	5.2 \pm 3.1%
6	27%	18%	30%	17%	15%	21.4 \pm 6.7%
7	11%	15%	13%	20%	4%	12.6 \pm 5.9%
8	35%	34%	35%	30%	12%	29.2 \pm 9.8%
9	23%	7%	11%	8%	6%	11.0 \pm 7.0%
10	25%	19%	17%	13%	25%	19.8 \pm 5.2%
11	7%	10%	12%	10%	11%	10.0 \pm 1.9%
12	54%	50%	61%	33%	44%	48.4 \pm 10.6%
13	21%	15%	14%	21%	19%	18.0 \pm 3.3%
14	18%	23%	7%	16%	16%	16.0 \pm 5.8%
15	12%	12%	8%	13%	11%	11.2 \pm 1.9%
16	30%	28%	19%	23%	23%	24.6 \pm 4.4%
17	6%	11%	10%	12%	12%	10.2 \pm 2.8%
18	36%	34%	26%	24%	22%	28.4 \pm 6.2%
19	20%	22%	20%	21%	10%	18.6 \pm 4.9%
20	10%	22%	18%	21%	12%	16.6 \pm 5.4%
21	37%	35%	31%	23%	27%	30.6 \pm 5.7%
22	12%	14%	10%	9%	10%	11.0 \pm 2.0%
23	14%	17%	8%	7%	20%	13.2 \pm 5.6%
24	14%	26%	24%	28%	21%	22.6 \pm 5.5%

S.D = standard deviation

Red shading = low-impulsive (lower quartile)

Green shading = high-impulsive (upper quartile)

Non-shaded = intermediate impulsive (middle half)

Table 2: Assignment of rats to impulsivity group based on the percentage of premature responses

Group	Mean \pm S.D	Range	Number of Rats
Low	9.77 \pm 2.29	(Range 5.20-11.20)	6
Medium	16.78 \pm 3.43	(Range 12-22.60)	12
High	33.30 \pm 8.72	(Range 24.60-48.40)	6

Coloured rows represent samples sequenced and validated by qRT-PCR, non-shaded samples were not analysed using RNA-Seq or qRT-PCR.

Table 3: Primers used for RNA Sequencing validation, (Figure 4)

Gene Name	Sequence Name
<i>Pnlsr</i>	Rn.PT.58.38329157
<i>Csmd3</i>	Rn.PT.58.34293471
<i>Mal</i>	Rn.PT.58.34591992
<i>Tspan2</i>	Rn.PT.58.34294959
<i>Ube3a</i>	Rn.PT.58.8938077
<i>Cplx1</i>	Rn.PT.58.34459545
<i>B2m</i>	Rn.PT.39a.22214834
<i>Actb</i>	Rn.PT.39a.22214838.g

The shaded rows represent the housekeeping genes.

Table 4: Exon Identification and number of altered exons in the NAcc of impulsive rats (Figure 5)

Ensembl Identification	Chromosome Location	Start	End	Total Exons	Exon Changes
ENSRNOG00000000483	20	5535434	5564657	20	1
ENSRNOG00000000940	12	9034308	9205905	30	1
ENSRNOG00000001068	12	13090372	13110592	8	1
ENSRNOG00000001404	12	22082057	22118220	15	1
ENSRNOG00000001516	3	58632476	58924038	35	1
ENSRNOG00000002021	11	31806618	31837763	13	2
ENSRNOG00000002207	14	62609484	62628862	17	1
ENSRNOG00000002270	14	11198896	11202669	11	1
ENSRNOG00000002827	10	8312961	8654892	17	1
ENSRNOG00000003905	10	91878633	92008082	23	1
ENSRNOG00000004180	7	66017086	66172360	4	1
ENSRNOG00000004208	6	788548	960703	17	1
ENSRNOG00000004483	7	59039720	59325947	16	1
ENSRNOG00000005345	6	37001356	37122023	4	2
ENSRNOG00000005504	3	45277348	45414613	21	1
ENSRNOG00000005927	7	60385688	60416925	10	2
ENSRNOG00000006426	7	50084060	50638798	13	1
ENSRNOG00000006905	6	72510255	72596446	43	1
ENSRNOG00000007256	5	28504558	28737719	13	1
ENSRNOG00000008169	5	105336262	105582375	13	1
ENSRNOG00000009042	15	4035196	4057157	29	1
ENSRNOG00000009960	7	117129237	117140214	15	1

ENSRNOG00000010103	7	143679617	143701452	17	1
ENSRNOG00000012237	8	56585396	56610612	19	1
ENSRNOG00000013659	10	94697016	94807499	12	1
ENSRNOG00000013781	9	27562959	27761733	16	1
ENSRNOG00000014576	8	115629583	115981910	54	1
ENSRNOG00000014637	7	126228295	126351634	12	1
ENSRNOG00000015018	3	113376983	113400707	13	1
ENSRNOG00000015528	9	111998203	112027240	11	2
ENSRNOG00000015967	5	152357270	152358643	3	1
ENSRNOG00000016271	15	55254706	55277713	6	2
ENSRNOG00000016670	17	85533057	85557939	5	1
ENSRNOG00000017155	9	50247692	50373284	5	1
ENSRNOG00000017824	3	161465743	161498951	8	1
ENSRNOG00000018225	3	150910398	150918525	4	1
ENSRNOG00000018406	3	60150021	60166013	8	1
ENSRNOG00000018651	17	5511385	5614435	31	1
ENSRNOG00000018873	1	165724451	165878652	9	1
ENSRNOG00000018988	9	100932545	100952416	12	1
ENSRNOG00000019768	16	8302950	8323293	11	1
ENSRNOG00000021202	1	222677365	222734241	11	1
ENSRNOG00000023338	2	205160405	205202764	8	1
ENSRNOG00000023861	8	94447550	94564525	33	2
ENSRNOG00000024852	3	127528434	127569093	8	1
ENSRNOG00000029360	20	38967238	38985036	10	1
ENSRNOG00000029441	16	26739389	26852179	15	1

Table 5: Functional Enrichment for genes altered in the NAcc of Impulsive Rats (Figure 3D)

Gene Ontology (GO) Annotation	P-value	Adjusted P-value	Z-score	Combined Score
sphingolipid catabolic process (GO:0030149)	0.000269	0.03	-3.34	27.46
sphingolipid metabolic process (GO:0006665)	0.000137	0.03	-3.01	26.77
regulation of stress fiber assembly (GO:0051492)	0.000322	0.03	-3.24	26.03
COPII vesicle coating (GO:0048208)	0.000547	0.03	-3.44	25.86
sphinganine-1-phosphate metabolic process (GO:0006668)	0.000182	0.03	-2.97	25.53
inositolphosphoceramide metabolic process (GO:0006673)	0.000182	0.03	-2.96	25.50
ceramide phosphoethanolamine metabolic process (GO:1905371)	0.000182	0.03	-2.96	25.47
sphingomyelin metabolic process (GO:0006684)	0.000244	0.03	-2.86	23.78
negative regulation of cell size (GO:0045792)	0.000826	0.03	-3.34	23.69
regulation of cell diameter (GO:0060305)	0.00051	0.03	-3.02	22.87
vascular endothelial growth factor receptor signaling pathway involved in lymphatic endothelial cell fate commitment (GO:0060851)	0.000482	0.03	-2.98	22.75

vascular endothelial growth factor receptor-3 signaling pathway (GO:0036325)	0.000482	0.03	-2.98	22.73
vascular endothelial growth factor receptor-1 signaling pathway (GO:0036323)	0.000482	0.03	-2.97	22.71
mitotic cell size control checkpoint (GO:0031567)	0.000443	0.03	-2.94	22.71
vascular endothelial growth factor receptor-2 signaling pathway (GO:0036324)	0.000524	0.03	-3.00	22.63

The z-score = the deviation from the expected rank by the Fisher exact test

Combined score = log of the p-value computed with the Fisher exact test multiplied by the z-score.

P values have been adjusted in consideration of multiple comparisons

Table 6: Functional Enrichment for genes with altered exon usage in the NAcc of Impulsive Rats (Figure 5D)

Gene Ontology (GO) Annotation	P-value	Adjusted P-value	Z-score	Combined Score
sphingolipid catabolic process (GO:0030149)	0.00027	0.03	-3.34	27.46
sphingolipid metabolic process (GO:0006665)	0.00014	0.03	-3.01	26.77
regulation of stress fiber assembly (GO:0051492)	0.00032	0.03	-3.24	26.03
COPII vesicle coating (GO:0048208)	0.00055	0.03	-3.44	25.86
sphinganine-1-phosphate metabolic process (GO:0006668)	0.00018	0.03	-2.97	25.53
inositolphosphoceramide metabolic process (GO:0006673)	0.00018	0.03	-2.96	25.50
ceramide phosphoethanolamine metabolic process (GO:1905371)	0.00018	0.03	-2.96	25.47
sphingomyelin metabolic process (GO:0006684)	0.00024	0.03	-2.86	23.78
negative regulation of cell size (GO:0045792)	0.00083	0.03	-3.34	23.69
regulation of cell diameter (GO:0060305)	0.00051	0.03	-3.02	22.87
vascular endothelial growth factor receptor signaling pathway involved in lymphatic endothelial	0.00048	0.03	-2.98	22.75

cell fate commitment (GO:0060851)				
vascular endothelial growth factor receptor-3 signaling pathway (GO:0036325)	0.00048	0.03	-2.98	22.73
vascular endothelial growth factor receptor-1 signaling pathway (GO:0036323)	0.00048	0.03	-2.97	22.71
mitotic cell size control checkpoint (GO:0031567)	0.00044	0.03	-2.94	22.71
vascular endothelial growth factor receptor-2 signaling pathway (GO:0036324)	0.00052	0.03	-3.00	22.63

The z-score = the deviation from the expected rank by the Fisher exact test

Combined score = log of the p-value computed with the Fisher exact test multiplied by the z-score.

P values have been adjusted in consideration of multiple comparisons

Table 7: Transcription Factors that Target significantly altered mRNA in the NAcc of Impulsive rats, (Figure 3E)

ENCODE and ChEA Consensus Transcription Factors from ChIP-X				
Transcription Factor Name, Data Source	Adjusted p-value	Z-score	Combined score	Genes*
UBTF_ENCODE	0.01012	-1.67	14.74	PAQR8;ZMYM2;HNRNPDL;UBE3A;NAA16;HCN2;GATA D2A;AES
TCF3_ENCODE	0.05095	-1.6	10.39	ZMYM2;CCNE2;CCNL1;JUN B;AES
PML_ENCODE	0.1105	-1.73	9.19	HNRNPDL;USP53;SNX14;UBE3A;CCNL1;JUNB
CREB1_CHEA	0.245	-1.5	6.32	HNRNPDL;USP53;CCNL1;JUNB;GATAD2A
KLF4_CHEA	0.245	-1.55	6.22	HNRNPDL;CCNL1;JUNB;GATAD2A
PBX3_ENCODE	0.3426	-1.57	5.02	ZMYM2;CCNL1;JUNB;GATAD2A
FOXA2_ENCODE	0.3426	-1.55	4.88	CCNE2;JUNB
NRF1_ENCODE	0.3426	-1.46	4.64	PNISR;CCNE2;SNX14;NAA16;GATAD2A
EZH2_ENCODE	0.3426	-1.52	4.6	CCDC39;HCN2
BCLAF1_ENCODE	0.3426	-1.56	4.44	SNX14;CCNL1;JUNB

P values have been adjusted in consideration of multiple comparisons ChIP = Chromatin

Immunoprecipitation, ChEA = ChIP Enrichment Analysis









































































* Genes in the gene set which bind with this transcription factor.

Table 8: Transcription Factors that Target significantly altered exon usage in the NAcc of Impulsive rats, (Figure 5E)

ENCODE and ChEA Consensus Transcription Factors from ChIP-X				
Transcription Factor Name, Data Source	Adjusted P- value	Z-score	Combined Score	Genes
RUNX1_CHEA	0.0004	-1.67	20.12	RBFOX1;FLT1;NCOA5;NCOA4;TEX2;PJA2;SNAP91;ING5;TMX4;SH3BGRL3;PKP4;TSPAN2;AGFG2
UBTF_ENCODE	0.0024	-1.64	15.70	PDIA3;RTN3;CPSF6;RDX;KLHL2;TEX2;SON;HECTD1;HNRNPDL;TP53INP2;PIP4K2A;RAC1;ITM2B
E2F6_ENCODE	0.0171	-1.59	11.50	RTN3;DOCK3;AGTPBP1;NCOA5;ING5;TMX4;SON;HECTD1;PUF60;HNRNPDL;PIP4K2A;PKP4;KCNQ5;SH3BGRL3;ATXN10;AGFG2;EIF4B
TCF7L2_ENCODE	0.0413	-1.68	10.19	PDIA3;TMX4;AGTPBP1;HECTD1;SEC24C;AGFG2
CREB1_CHEA	0.0826	-1.47	7.55	RBFOX1;SON;CPSF6;HNRNPDL;PUF60;TP53INP2;EIF4B;PJA2;AGFG2
SMAD4_CHEA	0.1379	-1.57	6.97	RBFOX1;DOCK3;FAM19A2;NCK2;AGFG2

P values have been adjusted in consideration of multiple comparisons, ChIP = Chromatin Immunoprecipitation, ChEA = ChIP Enrichment Analysis

Table 9: Percentile Rating of Rats in Additional Measure from the 2-CSRRT

Omissions		Accuracy		Latency	
1	 2.8	1	 98.6	1	 0.2918
2	 5.2	2	 95.2	2	 0.3832
3	 5.8	3	 95.3	3	 0.4043
4	 15.8	4	 86.1	4	 0.3361
5	 1.2	5	 98.7	5	 0.2852
6	 5.6	6	 93.8	6	 0.335
7	 13.6	7	 96.9	7	 0.3602
8	 5.8	8	 97.7	8	 0.3404
9	 5.0	9	 96.2	9	 0.308
10	 9.8	10	 95.3	10	 0.344
11	 2.8	11	 96.6	11	 0.2853
12	 12.6	12	 85.9	12	 0.441
13	 7.4	13	 94.0	13	 0.3141
14	 2.6	14	 96.4	14	 0.3082
15	 3.0	15	 96.7	15	 0.3614
16	 7.0	16	 90.6	16	 0.3155
17	 3.0	17	 95.3	17	 0.3423
18	 10.0	18	 93.7	18	 0.3398
19	 4.2	19	 96.8	19	 0.3402
20	 2.6	20	 97.8	20	 0.3342
21	 3.0	21	 91.9	21	 0.3127
22	 4.2	22	 94.9	22	 0.3158
23	 3.4	23	 97.6	23	 0.2752
24	 5.2	24	 91.7	24	 0.3713

Green Circle = high quartile, amber circle = intermediate half, red circle = low quartile.